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14. ABSTRACT

We hypothesize that BRCA2 facilitates the formation and/or the function of the ternary ISGF3 complex and thus, functional BRCA2 protein is essential for the antiproliferative effects of type I interferons against human breast tumor cells. S**pecific aims: (A)** To evaluate further the structural and functional interactions of BRCA2 with the members of the ISGF3 complex (STAT1, STAT2 and IRF9) in the human breast cells. **(B)** To evaluate the antiproliferative effects of BRCA2 over expression in the human breast cells with or without knock down of the IRF9 protein by RNA interference. **(C) To e**valuate the antiproliferative effects of type I interferons against tumors developed by BRCA2 positive and BRCA2 negative human breast tumor cells in the nude mice xenograft model.

15. SUBJECT TERMS
Breast cancer, BRCA2, Interferon alpha, Interferon beta, STAT1, STAT2, IRF9, ISGF3

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INTRODUCTION

BRCA2, a tumor suppressor whose inactivation is associated with hereditary breast and ovarian cancer predisposition, is essential for DNA repair in mammalian cells [1-5]. BRCA2-deficient cells are defective in the repair of DNA double-strand breaks by error-free homologous recombination [5, 6], allowing error-prone repair processes to create gross chromosomal re-arrangements that may promote carcinogenesis [6]. The role of BRCA2 in homologous recombination has been linked to its functions in the regulation of RAD51, a RecA-related recombinase that forms the nucleoprotein filaments on damaged DNA that are crucial to recombinational repair [7, 8]. BRCA2 binds directly to RAD51 through 6 of the 8 BRC repeats, ~30 amino acid motifs encoded within the central exon 11 region of all known mammalian BRCA2 genes [6, 9-12]. In addition to its role in DNA double-strand break repair, BRCA2 also plays a role in stabilization of stalled DNA replication forks, cytokinesis, transcription regulation, mammalian gametogenesis, centrosome duplication, and suppression of cell proliferation [6]. However, how BRCA2 mutations predispose women specifically to breast and ovarian cancer remains undefined. One of the possible pathways for the antiproliferative effect of BRCA2 is mediated through the MAGE-D1 protein [13]. BRCA2 binds and stabilizes MAGE-D1, a member of the MAGE gene family of proteins. Expression of BRCA2 and MAGE-D1 synergistically suppresses cell proliferation independently of the p53 pathway. MAGE-D1 is a downstream target of BRCA2 and that BRCA2 suppresses cell proliferation via stabilizing MAGE-D1 [13].

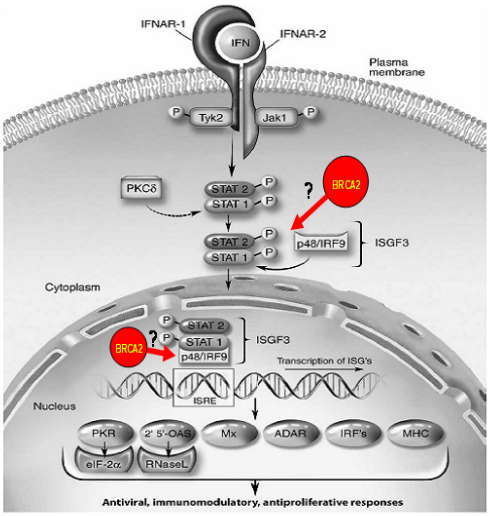

Fig. 1. Type I interferon (IFN) pathway (1-6). The postulated roles of BRCA2 (in red) are indicated.

We found that transient ablation of the breast cancer susceptibility gene BRCA2 in the human breast cells (Fig. 1) impairs the expressions of many type I interferon regulated genes (Table 1). Thus, it appears that type I interferons need functional BRCA2 for their actions [14]. Thus, negative regulation of IFN-induced genes in BRCA2-ablated breast cells may reflect another growth regulatory role of BRCA2.

The interferons (IFNs), in addition to their well-known antiviral activities, have important roles in the control of cell proliferation and are effective agents for the treatment of malignant diseases. IFNs not only regulate cell growth and division but also influence cell survival through their effects on apoptosis. α and β interferons are type I IFN proteins with antitumor activity [15, 16]. They down regulate oncogene expression and induce tumor suppressor genes, which result in antiproliferative activity. The classic pathway induced by type I IFNs involves the interaction of the IFN with two-receptor

subunits, IFNAR-1 and -2, which are associated with TYK-2 and JAK-1, respectively [17-19]. TYK-2 and JAK-1 phosphorylate tyrosine residues on the receptor that provide docking sites for the src-homology-2 (SH2) domains of STATs in a cell type specific manner [15, 16]. Once phosphorylated, STATs are released from the receptor and form heterodimers. In response to Type I IFNs, STAT2 is recruited to the IFNAR1 chain, where it is phosphorylated by TYK-2 and serves as a lure for STAT1 [15, 16]. Once released from the receptor, the resulting STAT1:STAT2 heterodimer associates with IRF9, a DNA binding protein (also called p48), forming a complex named IFN-stimulated gene factor-3 (ISGF3). After formation, ISGF3 translocates to the nucleus where it binds to the IFN-stimulated response elements (ISRE) upstream of IFN response genes and initiates transcription (Fig. 1).

We hypothesize that BRCA2 facilitates the formation and/or the function of the ternary ISGF3 complex and thus, functional BRCA2 protein is essential for the antiproliferative effects of type I interferons against human breast tumor cells.

Specific aims to verify the hypothesis are: (A) To evaluate further the structural and functional interactions of BRCA2 with the members of the ISGF3 complex (STAT1, STAT2 and IRF9) in the human breast cells. **(B)** To evaluate the antiproliferative effects of BRCA2 over expression in the human breast cells with or without knock down of the IRF9 protein by RNA interference. **(C)** To evaluate the antiproliferative effects of type I interferons against tumors developed by BRCA2 positive and BRCA2 negative human breast tumor cells in the nude mice xenograft model.

BODY

Task outlined in the approved Statement of Work for this period of the project

Task#1

To evaluate further the structural and functional interactions of BRCA2 with the members of the ISGF3 complex (STAT1, STAT2 and IRF9) in the human breast cells. (Months 1-18)

*We have little late start in full force because of the immigration delays to get the post-doctoral fellow. But we are catching up quickly and will finish the specific aims/tasks as proposed within the time period of funding.

1. We knocked down BRCA2 gene expression in the following human breast cancer cells: MCF7, MDA-MB-468, BT549, MDA-MB-231, T47D and MCF10A. We used Sigma Mission validated lentiviral shRNA particles to knockdown the BRCA2 mRNAs. The puromycin-resistant clones are evaluated for BRCA2 mRNA and protein by qRT-PCR (Applied Biosystems) and Western blotting analysis (R & D Systems), respectively. The BRCA2 mRNA levels decreased 70-85% (Fig. 1) and the protein levels decreased 55-65% (Fig. 2).

2. We evaluated the subcellular localization of ISGF3 complex in the control and BRCA2/KD cells. Our preliminary data suggested that BRCA2 binds with the

components of ISGF3. The first question we asked is whether the nuclear localization of ISGF3 is affected in BRCA2 deficiency. We determined the subcellular locations of ISGF3 components by immunofluorescence confocal microscopy. We used commercially available (Santa Cruz Biotech) antibodies against the ISGF3 component proteins for these experiments. For the detection of IRF9 we used ISGF-3γ p48 (B-18): sc-101070, a mouse monoclonal antibody. For Stat1 we used Stat1 p84/p91 (H-95) sc-98783 rabbit polyclonal antibody and for the detection of p-Stat1 we used p-Stat1 (A-2) sc-8394 mouse monoclonal antibody. For Stat-2 we used Stat-2 (H-190) sc-22816 rabbit polyclonal antibody and for p-Stat-2 we used p-Stat-2 (Tyr 690) sc-21689R rabbit polyclonal antibody. All these three proteins are found to be accumulated significantly in the cytosol in the BRCA2/KD cells as compared to the normal cells where these proteins are predominantly in the nucleus (Fig. 3). In the control cells, BRCA2 is co-localized in the nucleus with IRF9/Stat-1/Stat-2 complex, as expected. We used BRCA2 (I-17), an affinity purified goat polyclonal antibody raised against a peptide mapping at the N-terminus of BRCA2 of human origin for the immunofluorescence detection of the BRCA2 protein. We are evaluating whether phosphorylations of the STAT proteins (Tyr-701 in Stat-1β p84 and Stat1α p91 and Tyr-690 in Stat-2) are also required for their binding with BRCA2 by co-immunoprecipitation and Western blotting with phospho-specific antibodies. We are expressing N-terminal GST-tagged Stat-2 protein or Stat-2 protein with mutation (Tyr-Ala) at the Tyr-690 in breast cells like MCF7 and T47D. The Stat-2 protein without Tyr-690 will not be able to be phosphorylated. We will evaluate whether this protein can form complex with BRCA2.

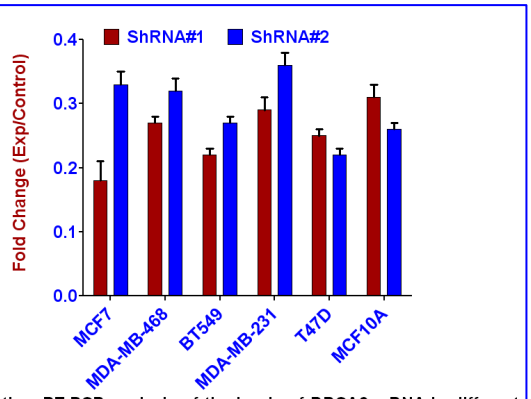

Fig. 1. Real time RT-PCR analysis of the levels of BRCA2 mRNA in different human breast cells after knockdown of the BRCA2 mRNA with two different shRNAs. Scrambled shRNA was used as control. Results are Mean + SEM (n=6). Difference in the levels of BRCA2 mRNAs with either shRNA#1 or shRNA#2 were statistically significant (p<0.001).

3. We have developed a BRCA2 derivative that lacks only the nuclear localization signals but not the other C-terminal domains. We started with the pCINBRCA2WT plasmid, a clone of full-length human BRCA2 protein in pcDNA3 vector, a gift from Prof. Mien-Chie Hung of MD Anderson. We designed primers to amplify the whole plasmid except the two nuclear localization signals at the C-terminus of the BRCA2 cDNA following long-distance PCR protocols. We introduced stop codon and Fse I (NEB) site in the primers. Fse I site is absent in the vector and the insert DNA. We

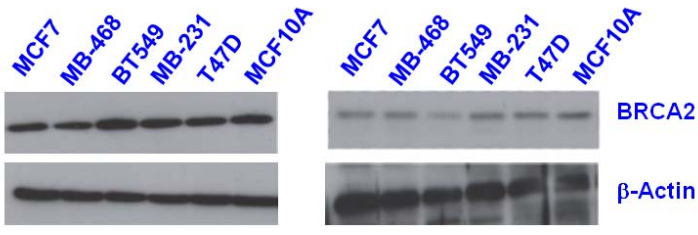

Fig. 2. Western blot analysis for the relative levels of BRCA2 protein in different human breast cells lines before and after shRNA-mediated knockdown of this mRNA in these cells. β-Actin was used as loading controls.

digested the purified amplified DNA with Fse I and relegated the plasmid DNA. This plasmid expressed a C-terminal 153 amino acid residue truncated BRCA2 protein lacking the two nuclear localization signals (Fig. 4). We expressed this NLS-deleted version of BRCA2 in Capan-1 cells and currently characterizing the recombinant clones. We will employ these recombinant cells to further verify the need for nuclear localization of the BRCA2 protein for the activation of the ISG15 gene promoter. We will also use these cells for subcellular fractionation and co-immunoprecipitation to evaluate by Western blotting whether BRCA2-ISGF3 complex is formed in the cytosol. These data will be generated soon.

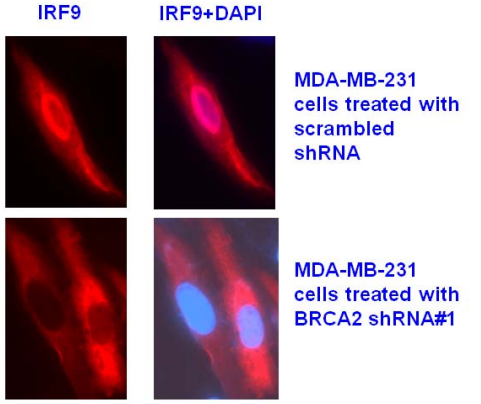

Fig. 3. Representative fluorescent micrograph showing significant decrease in the nuclear localization of IRF9 in BRCA2 knocked down MDA-MB-231 cells. Similar results were obtained with other breast cells tested and with STAT proteins.

Ongoing experiments:

1. We are preparing for the evaluation what the domains of human BRCA2 protein that are essential for the BRCA2/ISGF3 complex formation. Human BRCA2 is a large protein (3418 amino acids). We have amplified 5 consecutive fragments from the human BRCA2 ORF (in plasmid pCINBRCA2WT). There will be five peptide fragments the first 4 fragments from the N-terminal end will have 683 amino acid (ORF ~2055 bp with start and stop codons) and the 5th C-terminal fragment will have 686 amino acids. We have cloned these PCR amplified products into N-terminal GST-tagged protein expression vector (pFN2K (GST) Flexi® Vector). We will express these N-terminal GST-tagged BRCA2 peptide fragments in human breast cells. By GST-pull down assays we will determine which domains of the human BRCA2 protein are required for the complex formation with ISGF3 components.

MPIGSKERPTFFEIFKTRCNKADLGPISLNWFEELSSEAPPYNSEPAEESEHKNNNYEPNLFKTPQRKPSYNQLAST
PIIFKEQGLTLPLYQSPVKELDKFKLDLGRNVPNSRHKSLRTVKTKMDQADDVSCPLLNSCLSESPVVLQCTHVTPQ
RDKSVVCGSLFHTPKFVKGRQTPKHISESLGAEVDPDMSWSSSLATPPTLSSTVLIVRNEEASETVFPHDTTANVKS
YFSNHDESLKKNDRFIASVTDSENTNQREAASHGFGKTSGNSFKVNSCKDHIGKSMPNVLEDEVYETVVDTSEEDSF
SLCFSKCRTKNLQKVRTSKTRKKIFHEANADECEKSKNQVKEKYSFVSEVEPNDTDPLDSNVANQKPFESGSDKISK
EVVPSLACEWSQLTLSGLNGAQMEKIPLLHISSCDQNISEKDLLDTENKRKKDFLTSENSLPRISSLPKSEKPLNEE
TVVNKRDEEQHLESHTDCILAVKQAISGTSPVASSFQGIKKSIFRIRESPKETFNASFSGHMTDPNFKKETEASESG
LEIHTVCSQKEDSLCPNLIDNGSWPATTTQNSVALKNAGLISTLKKKTNKFIYAIHDETSYKGKKIPKDQKSELINC
SAQFEANAFEAPLTFANADSGLLHSSVKRSCSQNDSEEPTLSLTSSFGTILRKCSRNETCSNNTVISQDLDYKEAKC
NKEKLQLFITPEADSLSCLQEGQCENDPKSKKVSDIKEEVLAAACHPVQHSKVEYSDTDFQSQKSLLYDHENASTLI
LTPTSKDVLSNLVMISRGKESYKMSDKLKGNNYESDVELTKNIPMEKNQDVCALNENYKNVELLPPEKYMRVASPSR
KVQFNQNTNLRVIQKNQEETTSISKITVNPDSEELFSDNENNFVFQVANERNNLALGNTKELHETDLTCVNEPIFKN
STMVLYGDTGDKQATQVSIKKDLVYVLAEENKNSVKQHIKMTLGQDLKSDISLNIDKIPEKNNDYMNKWAGLLGPIS
NHSFGGSFRTASNKEIKLSEHNIKKSKMFFKDIEEQYPTSLACVEIVNTLALDNQKKLSKPQSINTVSAHLQSSVVV
SDCKNSHITPQMLFSKQDFNSNHNLTPSQKAEITELSTILEESGSQFEFTQFRKPSYILQKSTFEVPENQMTILKTT
SEECRDADLHVIMNAPSIGQVDSSKQFEGTVEIKRKFAGLLKNDCNKSASGYLTDENEVGFRGFYSAHGTKLNVSTE
ALQKAVKLFSDIENISEETSAEVHPISLSSSKCHDSVVSMFKIENHNDKTVSEKNNKCQLILQNNIEMTTGTFVEEI
TENYKRNTENEDNKYTAASRNSHNLEFDGSDSSKNDTVCIHKDETDLLFTDQHNICLKLSGQFMKEGNTQIKEDLSD
LTFLEVAKAQEACHGNTSNKEQLTATKTEQNIKDFETSDTFFQTASGKNISVAKESFNKIVNFFDQKPEELHNFSLN
SELHSDIRKNKMDILSYEETDIVKHKILKESVPVGTGNQLVTFQGQPERDEKIKEPTLLGFHTASGKKVKIAKESLD
KVKNLFDEKEQGTSEITSFSHQWAKTLKYREACKDLELACETIEITAAPKCKEMQNSLNNDKNLVSIETVVPPKLLS
DNLCRQTENLKTSKSIFLKVKVHENVEKETAKSPATCYTNQSPYSVIENSALAFYTSCSRKTSVSQTSLLEAKKWLR
EGIFDGQPERINTADYVGNYLYENNSNSTIAENDKNHLSEKQDTYLSNSSMSNSYSYHSDEVYNDSGYLSKNKLDSG
IEPVLKNVEDQKNTSFSKVISNVKDANAYPQTVNEDICVEELVTSSSPCKNKNAAIKLSISNSNNFEVGPPAFRIAS
GKIVCVSHETIKKVKDIFTDSFSKVIKENNENKSKICQTKIMAGCYEALDDSEDILHNSLDNDECSTHSHKVFADIQ
SEEILQHNQNMSGLEKVSKISPCDVSLETSDICKCSIGKLHKSVSSANTCGIFSTASGKSVQVSDASLQNARQVFSE
IEDSTKQVFSKVLFKSNEHSDQLTREENTAIRTPEHLISQKGFSYNVVNSSAFSGFSTASGKQVSILESSLHKVKGV
LEEFDLIRTEHSLHYSPTSRQNVSKILPRVDKRNPEHCVNSEMEKTCSKEFKLSNNLNVEGGSSENNHSIKVSPYLS
QFQQDKQQLVLGTKVSLVENIHVLGKEQASPKNVKMEIGKTETFSDVPVKTNIEVCSTYSKDSENYFETEAVEIAKA
FMEDDELTDSKLPSHATHSLFTCPENEEMVLSNSRIGKRRGEPLILVGEPSIKRNLLNEFDRIIENQEKSLKASKST
PDGTIKDRRLFMHHVSLEPITCVPFRTTKERQEIQNPNFTAPGQEFLSKSHLYEHLTLEKSSSNLAVSGHPFYQVSA
TRNEKMRHLITTGRPTKVFVPPFKTKSHFHRVEQCVRNINLEENRQKQNIDGHGSDDSKNKINDNEIHQFNKNNSNQ
AAAVTFTKCEEEPLDLITSLQNARDIQDMRIKKKQRQRVFPQPGSLYLAKTSTLPRISLKAAVGGQVPSACSHKQLY
TYGVSKHCIKINSKNAESFQFHTEDYFGKESLWTGKGIQLADGGWLIPSNDGKAGKEEFYRALCDTPGVDPKLISRI
WVYNHYRWIIWKLAAMECAFPKEFANRCLSPERVLLQLKYRYDTEIDRSRRSAIKKIMERDDTAAKTLVLCVSDIIS
LSANISETSSNKTSSADTQKVAIIELTDGWYAVKAQLDPPLLAVLKNGRLTVGQKIILHGAELVGSPDACTPLEAPE
SLMLKISANSTRPARWYTKLGFFPDPRPFFLPLSSLFSDGGNVGCVDVIIQRAYPIQWMEKTSSGLYIFRNEREEEK
EAAKYVEAQQKRLEALFTKIQEEFEEHEENTTKPYLPSRALTRQQVRALQDGAELYEAVKNAADPAYLEGYFSEEQL
RALNNHRQMLNDKKQAQIQLEIRKAMESAEQKEQGLSRDVTTVWKLRIVSYSKKEKDSVILSIWRPSSDLYSLLTEG
KRYRIYHLATSKSKSKSERANIQLAATKKTQYQQLPVSDEILFQIYQPREPLHFSKFLDPDFQPSCSEVDLIGFVVS
VVKKTGLAPFVYLSDECYNLLAIKFWIDLNEDIIKPHMLIAASNLQWRPESKSGLLTLFAGDFSVFSASPKEGHFQE
TFNKMKNTVENIDILCNEAENKLMHILHANDPKWSTPTKDCTSGPYTAQIIPGTGNKLLMSSPNCEIYYQSPLSLCM
AKRKSVSTPVSAQMTSKSCKGEKEIDDQKNCKKRRALDFLSRLPLPPPVSPICTFVSPAAQKAFQPPRSCGTKYETP
IKKKELNSPQMTPFKKFNEISLLESNSIADEELALINTQALLSGGSTGEKQFISVSESTRTAPTSSEDYLRLKRRCTT
SLIKEQESSQASTEECEKNKQDTITTKKYI

Fig. 4. Amino acid sequence of human BRCA2 protein showing (highlighted in yellow) the 153 amino acid nuclear localization signals that we have deleted.

2. Further evaluation of direct binding between BRCA2 and ISGF3 components by yeast 2-hybrid analysis. We are using BD Clontech Matchmaker Gold yeast two hybrid system for this purpose. We have cloned the five BRCA2 ORF fragments described in the previous section into pGBKT7 DNA-BD cloning vector. Similarly we have cloned IRF9 and Stat-2 (wild type and Y690A mutant) ORFs into pGADT7 AD cloning vector. We are also cloning Stat-1 ORFs into pGADT7 vector. We should be able to determine which domain of BRCA2 directly interacts with these proteins using this yeast two hybrid system, within next 6 months.

Key Research Accomplishments

- We knocked down BRCA2 gene expression in the following human breast cancer cells: MCF7, MDA-MB-468, BT549, MDA-MB-231, T47D and MCF10A.
- We evaluated the subcellular localization of ISGF3 complex in the control and BRCA2/KD cells.
- We have developed a BRCA2 derivative that lacks only the nuclear localization signals but not the other C-terminal domains.
- We are preparing for the evaluation what the domains of human BRCA2 protein that are essential for the BRCA2/ISGF3 complex formation by Co-IP and yeast 2-hybrid analyses.

REPORTABLE OUTCOMES: We have not yet published or presented the research performed in this grant project. But the research performed in this project directly or indirectly contributed to the following publications and poster abstracts.

Publication:

[1] Mittal, M., Myers, J. N., Misra, S., Bailey, C. K. and **Chaudhuri, G.** (2008) In vivo binding to and functional repression of the VDR gene promoter by SLUG in human breast cells. ***Biochem. Biophys. Res. Commun***. **372**, 30-34.

[2] Mittal, M. K., Myers, J. N., Bailey, C. K., Misra, S. and **Chaudhuri, G.** (2009) Mode of action of the retrogene product SNAI1P, a SNAIL homolog, in human breast cancer cells. ***Mol. Biol Report*** (In press) DOI 10.1007/s11033-009-9492-8.

Meeting abstracts:

We are planning to submit an abstract on the research progress we have made in this subject to the AACR sponsored **Frontiers in Basic Cancer Research** meeting to be held in Boston, MA on October 8-11, 2009.

The current research on this project directly or indirectly affected the studies performed in the following poster presentations from our lab during 2008-2009:

[1] Mittal, Mukul K., and **Chaudhuri, G.** (2008) ChIP-DSL technology reveals an extensive SLUGbinding program on human gene promoters in breast cells. Presented as a poster at the fifth Era of Hope Meeting on June 25—June 28, 2008, at the Baltimore Convention Center in Baltimore, Maryland.

[2] Chaudhuri, G. and Misra, Smita, (2008) Mechanisms of silencing and desilencing of BRCA2 gene expression in human breast cells. Presented as a poster at the fifth Era of Hope Meeting on June 25—June 28, 2008, at the Baltimore Convention Center in Baltimore, Maryland.

[3] Myers, J. N., Mittal M., and Chaudhuri, G. (2008) *In vivo* Binding to and functional repression of the VDR gene promoter by SLUG in human breast cancer. Poster presented at the American Society of Hematology 50th Annual Meeting and Exposition Conference, San Francisco CA. December 6-9, 2008.

[4] Bailey, Charvann K., Mittal, M. K., Misra, S., and **Chaudhuri, G.** (2009) The mechanism of SLUG-CtBP1 interactions on the E2-box of DNA *in vivo.* Presented as poster at the AACR meeting in Denver, CO in April 18-22, 2009.

[5] Booker, B, Komangoye, R., Bailey, Charvann K., Mittal, M. K., Misra, S., and **Chaudhuri, G**. (2009) Mutational characterization of the C-terminal zinc finger of human SNAIL protein. Presented as poster at the AACR meeting in Denver, CO in April 18-22, 2009.

[6] Komangoye, R., Booker, B, Bailey, Charvann K., Mittal, M. K., Misra, S., and **Chaudhuri, G.** (2009) Development of an artificial zinc finger protein against human SNAIL protein. Presented as poster at the AACR meeting in Denver, CO in April 18-22, 2009.

[7] Misra, S.**,** and **Chaudhuri, G.** (2009) Peroxiredoxin 5 as a mediator for de-silencing of BRCA2 gene expression in dividing human breast cells. Presented as poster at the AACR meeting in Denver, CO in April 18-22, 2009.

[8] Misra, S.**,** and **Chaudhuri, G.** (2009) Regulation of the bi-directional promoter of human BRCA2/ZAR2 genes by folate-dependent DNA methylation. Presented as poster at the AACR meeting in Denver, CO in April 18-22, 2009.

[9] Mittal, M. K., Singh, K. and **Chaudhuri, G.** (2009) Mechanisms of SLUG-induced drug resistance development in breast cancer cells. To be presented as poster at the 2009 San Antonio Breast Cancer Symposium (SABCS) to be held in Henry B. Gonzalez Convention Center, San Antonio, Texas, USA, December 9-13, 2009.

Conclusion:

ISGF3 complex is formed in the cytosol with the help of the BRCA2 protein and this complex is largely retained in the cytoplasm when BRCA2 is knocked down in the human breast cells. Further analysis id going on to understand the domains of BRCA2 protein that are required for ISGF3 complex formation.

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APPENDICES: None.